

JG12 9484 PCT/PTO 18 JUN 2001

09/868463

THE COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

DOCKET NO.998-021
Date: June 18, 2001

Sir:

Transmitted herewith for filing Under 35 U. S. C. 371, (PCT national phase) is the Patent Application of:
Inventor: Diem; Max , Bargonetti; Jill , Gopen; Tamara , Boydston-White; Susie

For: Method of Characterization of Biological Entities

The present application claims priority date of US Provisional application 60/116,755 , filed 22 January, 1999, and PCT /US00/01550 filed 22 January, 2000.

The filing fee has been calculated as shown below:

| | Col. 1 | Col. 2 | SMALL ENTITY FEE | |
|---|-----------|-----------|------------------|-----|
| FOR: | NO. FILED | NO. EXTRA | RATE | FEE |
| BASIC FEE | | | | 355 |
| TOTAL CLAIMS | 38-20 | 18 | X 9 | 162 |
| INDEP. CLAIMS | 4-3 | 0 | X40 | 40 |
| ___ MULTIPLE DEPENDENT CLAIM PRESENTED | | | | |
| If the difference in Col. 1 is less than 0, enter "0" in Col 2. | | | | |
| TOTAL | | | | 557 |

June 18, 2001
Date


Signature

Rodney T. Hodgson, Agent No. 37849
Typed or printed name

EXPRESS MAIL CERTIFICATE

"Express Mail" label number: EK956203701US

Date of Deposit: June 18, 2001

I hereby certify that the following **attached** paper or fee

Patent Application (20 Pages specification and claims)

7 sheets drawings

Declaration and Power of Attorney (2 pages)

Patent Transmittal Sheet

Check for \$597 to the Commissioner of Patents and Trademarks

Deposit Account Authorization form (2 copies))

Assignment (3 pages)

Recordation form

Acknowledgment Card

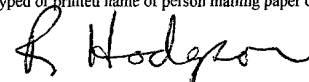
is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.



24295

PATENT TRADEMARK OFFICE

Rodney T. Hodgson Agent No 37849
(Typed or printed name of person mailing paper or fee)



(Signature of person mailing paper or fee)

For: Method of Characterization of Biological Entities

Filed 22 Jan 2000

Inventor: Diem; Max , Bargonetti; Jill , Gopen; Tamara , Boydston-White; Susie , ,

Serial No.: PCT 00/01550

DEPOSIT ACCOUNT AUTHORIZATION

Please charge Deposit Account No. 08-2240 in the amount of \$0.00. A duplicate copy of this sheet is enclosed.


The Commissioner is hereby authorized to charge payment of the following fees except issue fee or credit any overpayment to Deposit Account No. 08-2240 regarding the above identified application.

Any additional filing fees required.

Any patent application processing fees.

A duplicate copy of this sheet is enclosed.

Respectfully submitted,

By 
Agent: Rodney T. Hodgson
Registration No.: 37,849
Tel. (914) 762-5248
FAX (914) 762-4126

Rodney T. Hodgson, Ph.D.
822 Pinesbridge Road,
Ossining, NY 10562_



24295

PATENT TRADEMARK OFFICE

7/8/95

09/868463

JCO3 Rec'd PGT/PTC

18 JUN 2001

1

1

Method of Characterization of Biological Entities

FIELD OF THE INVENTION

The field of the invention is the field of infrared spectra of biological entities such as single exfoliated cells from normal and abnormal patients. Differences in such spectra can be used to detect cancer in samples of cells and tissues, and can be used as a screening test.

6

RELATED PATENTS AND APPLICATIONS

This application claims priority from a provisional application 60/33,529 filed 1/22/99 entitled "A system and method to determine the absence or presence of cancerous disease by infrared spectroscopy", by Diem et al.

11

BACKGROUND OF THE INVENTION

Previous papers and patents claimed to be able to detect the differences between normal and abnormal (pre-cancerous and cancerous) cells and tissue by inspection of the infrared spectra of these cells and tissues. Although some of these patent applications and scientific reports present at least partially valid data, the interpretation of these data mostly lacks the specific understanding of the origin of spectral differences between normal and abnormal cells and tissues.

Early patent applications and scientific reports by Wong and coworkers were based on faulty interpretation of spectral differences in cervical and other cells and tissues. These studies failed to take into account the spectral changes in cells and tissues associated with maturation and differentiation of cells. Since certain cancerous and pre-cancerous diseases are accompanied by disruptions of the regular maturation of cells in tissues, some weak correlation between cancerous disease and spectral features was observed. The inconsistencies of the correlations were blamed on failures of standard methods of cytology and pathology.

Although some of the shortcomings of the earlier patents had been established, US Patent # 5,733,739 which amplifies the misinterpretations of earlier reports and patents, and uses data that are clearly misinterpreted, has issued. For example, the patent used infrared (IR) spectral data from extracellular materials, such as mucus, and other confounding factors such as blood cells, for the interpretation of the spectral characteristics of cervical cells. Thus, most data used in their patent are unrelated to actual cancerous and pre-cancerous disease but rather to gross spectral changes due to contamination of cervical cells. The actual spectral changes due to cancerous disease, to be

1 discussed below, cannot be detected by the crude methods described in patent No. 5,733,739.

US Patent 5,596,992 uses infrared spectroscopy to distinguish normal from cancerous leukocytes and other cells by multivariate statistical methods. These studies use highly homogeneous samples and, therefore, have a much higher success in predicting disease from infrared data. However, they have failed to realize a source of spectral heterogeneity that confounds
6 the interpretation of the data, and is due to the stages of cells' reproductive cycle.

We have established that identical and highly pure cells still present spectral heterogeneity due to the differences in their development. Only when cells are separated into homogeneous fractions according to their stage in the cell cycle will homogeneous spectral patterns be observed. Under these circumstances, single cells in one given stage exhibit spectral characteristics that can
11 be directly related to the presence of cancer. Thus, the understanding of the cellular biology underlying the cell's reproductive cycle is necessary for a reliable diagnosis of disease. A method will be reported here that allows the detection of single cells that carry the signature of cancerous disease.

OBJECTS OF THE INVENTION

16 It is an object of the invention to provide an apparatus and a method for determining characteristics of large numbers of biological entities such as cells.

It is an object of the invention to distinguish normal from abnormal cell populations by statistical analysis of characteristics of a large number of single cells or other entities.

It is an object of the invention to provide an apparatus for measuring the infrared vibrational
21 spectrum of large numbers of single cells or other entities.

It is an object of the invention to provide an apparatus which measures the infrared vibrational spectrum of such a large number of cells or entities that meaningful statistical analysis is possible, in a in a time short enough that the process may be carried out at low cost.

SUMMARY OF THE INVENTION

26 Apparatus and a method for using the apparatus to determine the infrared vibrational spectral absorption of a large number of individual cells or other biological entities is disclosed.

The infrared vibrational spectra characterizing the presence of DNA in the cells is used to determine the statistical proportion of the cells in a non quiescent state, the that proportion is used to determine if the cells represent a cell population having cancerous or other anomalous
31 characteristics.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts schematically the stages of the division cycles somatic cells undergo. In cancerous cell cultures, cells often cycle directly from one division cycle into the next; *i.e.*, they are dividing constantly. The width of each slice shown is approximately proportional to the percentage of time the cells spend in a given stage.

Figure 2A shows a typical infrared absorption spectrum of a protein film. The peaks are referred to by standard nomenclature of vibrations giving rise to the absorptions. Figure 2B and Fig. 2C show the infrared absorption spectra of DNA and RNA respectively. The horizontal axis (abscissa) describes the wavelength of the infrared radiation, expressed in units of inverse length. The ordinate denotes the amount of light absorbed by a vibration (absorbance) and is presented in arbitrary units. Single cells typically exhibit between 0.05 and 0.2 absorbance units.

Figure 3 shows the infrared traces of DNA and RNA, each superimposed on a protein background spectrum. Protein and nucleic acid intensities are adjusted such that their intensities correspond approximately to the intensity ratios observed in cells and tissues. Note that the resulting spectral composites can be easily distinguished; thus, the spectral patterns in Figure 3 can be used to judge whether DNA or RNA constitutes most of the observable nucleic acid in a cell or tissue.

Figure 4 shows the different spectral traces observed for cultured cells separated into different phases of their division cycle by elutriation. Trace A shows the averaged spectrum obtained for an exponentially growing cell culture, that is, a mixture of all cell division phases. Trace B shows a spectrum for cells in the G1 phase, trace C in the early S phase, trace D in the late S phase and trace E in the G2 phase. Note the differences in the amount of DNA observable between G1 and G2 phase on one hand, and the S phase on the other.

Figure 5 shows a comparison between normal and abnormal squamous cells, clustered by cell cycle phase. In Figure 5A, the traces of normal and abnormal cells are virtually indistinguishable, indicating that some cells in a sample of abnormal cells maintain normal spectral properties. We attribute these spectral patterns to be associated with inactive (non-dividing) cells of the G0 phase. The spectral traces in Figure 5B are believed to be due to the G1 phase. The traces due to abnormal cells agree well with those of the G1 trace observed in Figure 4, trace B, for cancerous ML-1 cells. We believe that the "normal" trace in Figure 5B differs from the abnormal ones by less DNA spectral contributions. This view is justified by the strong DNA features at 1230 cm^{-1} , and the small DNA shoulder at 970 cm^{-1} in the "abnormal" spectra.

1 Figure 6 shows a microscopic image taken of Eu^{3+} stained cells through a fluorescence microscope. The arrow indicates an approximate scale of the display. The Figure demonstrates that excellent separation of cells is easily achieved, and that cells can be localized by their fluorescent emission. This localization of cells is used to speed up data manipulation for the infrared imaging, and may serve as indications whether or not single cells are in the field of view of a given infrared
6 detector element.

Figure 7 shows a sketch of the most preferred embodiment of the invention.

Figure 8 shows a sketch of a preferred embodiment of the invention.

Figure 9 shows a sketch of a preferred embodiment of the invention.

11 DETAILED DESCRIPTION OF THE INVENTION

SCIENTIFIC ASPECTS OF THE INVENTION

a) Aspects of Cellular Biology

Mammalian and other cells undergo division according to a cell cycle scheme depicted in Figure 1. Normal cells are found predominantly in a state, referred to as G_0 , in which no
16 reproduction occurs. The division process is initiated by certain biochemical signals, upon which a number of predetermined processes occur that causes a cell to duplicate itself. This duplication process may take 20 to 30 hours, and can be divided into phases Gap 1 (G_1), Synthesis (S), Gap 2 (G_2) and Mitosis (M). In these phases, well-established processes take place: for example, in the S phases, the DNA strands containing the genetic blueprint of the cell are duplicated, whereas in
21 the M phase, the actual cell division takes place; *i.e.*, two new cells are created from the progenitor cell.

Cancerous cells can be cultured indefinitely if the cells are kept at proper conditions for cell growth. In such cell cultures, the cells re-enter the next division cycle as soon as the previous one is completed, and the number of cells doubles after a period of time corresponding in length to the
26 cell division cycle. Such a cell culture is said to be exponentially growing, and the cells found in a given phase of the cycle, *i.e.* G_1 , S, G_2 and M, is determined by the relative length of these stages, which is about 10 hours for the S phase, 8 hours for G_1 , 6 hours for G_2 , and minutes for M.

In order to obtain cells at given stages of their cycles, cultured cells can be separated into
31 fractions of good phase homogeneity by a density/size centrifugation called elutriation.

- 1 Subsequently, fluorescence activated cell sorting (FACS) technology can be used to identify these fractions as G1, S or G2 phases by the DNA content. These methods present the opportunity to interpret the changes in infrared spectra of cells at various stages of their cell cycles.

b) Aspects of Vibrational Spectroscopy

- In infrared spectroscopy, the attenuation of the intensity of a beam of infrared light upon
 6 passing through a sample is measured. This attenuation is caused by the interaction of the light with the vibrational transitions of the sample molecules. These absorptions of infrared light, when plotted against the wavelength of the light, produce a unique fingerprint pattern of the molecules encountered by the beam of light. The fingerprint pattern is very useful in identifying entities of biological interest, which include but are not limited to cells, proteins, viruses, fluids, etc. Such
 11 fingerprint patterns for a number of cellular components are shown later in this specification. In addition, one can assess the degree of packing of certain cellular components from the infrared spectral patterns: *i.e.*, we have demonstrated before that a nucleus of a quiescent cell is optically so dense that it may not transmit any of the incident infrared radiation, and hence no infrared spectral features due to the cell nucleus are measurable. Consequently, infrared spectroscopy and
 16 the appearance of spectral feature due to the nucleus may be used to monitor nuclear processes which result in significant changes in the packing of the constituent molecules.

- In infrared microspectroscopy (also referred to as infrared microscopy) the infrared beam is passed through the specimen and focused by an infrared microscope that allows infrared spectral data to be collected from microscopic particles, such as single cells, or pixels of tissue the size of
 21 a cell.

- The variety of molecules found in a human cell is so staggering that the unambiguous assignment of the infrared spectra of a cell's constituents is not possible, particularly since different proteins, in general, have similar vibrational spectra. However, we and others have shown in the past that changes in molecular composition can be observed and interpreted reliably. For example,
 26 different protein/nucleic acid ratios, or the overexpression of structural proteins, can be monitored via infrared microspectroscopy.

c) Aspects of Infrared Spectra of Cells and Tissue

- The following section presents a detailed view of recent progress in understanding the infrared spectroscopy of cells and tissues. Such a discussion, and the detailed understanding, has
 31 been absent in many of the previous publications and patent applications. Consequently, these

1 earlier efforts were based on intuition, rather than scientific results, and reached unfounded claims and conclusions.

We have found that the infrared spectra of cells and tissues are dominated by the infrared spectral patterns of proteins (as shown in Fig. 2 trace A), which constitute the predominant cellular component by mass. Although there are hundreds or thousands of different proteins in a cell, the protein spectrum observed is determined by the most abundant proteins which generally are structural proteins such as tubuline that determine a cell's overall shape and physical properties.

Protein infrared absorption spectra are known to vary with the secondary structure of the protein (α -helical, pleated sheet, random, *etc.*), the protein's state of hydration, the solvent's ionic strength, *etc.* However, the averaged infrared spectra of all metabolic and structural proteins found in cells turn out to be remarkably the same for most cells. The only proteins that exhibit distinctly different spectra are found in connective tissue (*e.g.*, collagen).

The averaged protein spectra found inside cells are dominated by the amide I band at *ca.* 1650 cm^{-1} , the amide II vibration at 1530 cm^{-1} , the amide III peak at 1245 cm^{-1} , and a number of side chain vibrations in the 1310 , 1390 and 1450 cm^{-1} range.

16 The infrared absorption spectra of RNA and DNA are shown in Figure 2, Trace B and C, respectively. These spectra exhibit absorption peaks between 1580 and 1700 cm^{-1} due to the aromatic base breathing and $\text{C}=\text{O}$ stretching vibrations. The ionized PO_2^- and ribose groups exhibit a triad of peaks that occur in DNA at 1071 , 1084 and 1095 cm^{-1} with nearly equal intensities. Further DNA peaks are observed at 965 and 1245 cm^{-1} (the phosphodiester vibration). In RNA, 21 the peak at 1085 cm^{-1} is stronger than the two other peaks in this triad, and forms a distinct "nose". Furthermore, the intensity ratio of this triad to the phosphodiester peak at 1245 cm^{-1} is about $1:0.7$, whereas it is about unity in DNA. Since nucleic acid vibrational spectra in cells in tissues are always observed in the presence of protein, we show in Figure 3 expanded regions of the DNA and RNA spectra superimposed on protein spectra.

26 In cells, DNA is found in the nucleus and in mitochondria. RNA, on the other hand, can occur in the cytoplasm as ribosomal or transfer RNA (r-RNA and t-RNA), and in the nucleus and cytoplasm as messenger RNA (m-RNA). Thus, the cytoplasm is relatively rich in various RNA species, whereas the nucleus contains nearly all the DNA.

Terminally differentiated, and no longer proliferative cells exhibits virtually no DNA 31 spectral features. Consequently, in these cells mainly the cytoplasmic RNA spectral features are observed superimposed on the protein spectrum. In such cells, the nucleus is very small and very well delimited. The concentration of DNA, RNA and protein in such a nucleus is quite high, and

1 leads to an optical density of the nucleus in excess of 1 absorbance unit. However, DNA is not distributed uniformly throughout the nucleus; rather, it is wrapped tightly around proteins known as histones. Thus, the local concentration of DNA and protein is even higher, and it is likely that the chromosomes of an inactive nucleus will appear as "black" (*i.e.*, opaque or nearly opaque) strings. Little or no spectral information from the DNA can be collected in this case.

6 Benedetti *et al.* presented spectral results that confirm this hypothesis. They have observed cells in which there were 64 copies of nuclear DNA whereas in normal cells, there are two copies of DNA. The cells with higher DNA content do not exhibit DNA spectral features stronger than those observed in cells with two copies. Interestingly, with the onset of pre-cancerous and cancerous disease, the DNA signals become generally more pronounced. This aspect, and models
11 to explain it, will be discussed later in this application.

Aside from protein and nucleic acids, carbohydrates (in the form of polymeric sugars or glycoproteins), phospholipids, water, and a few other compounds may occur in cells and tissues at levels that make them detectable *via* infrared spectroscopy.

d) Aspects of Infrared Spectra at various Cell Cycle Stages

16 Even cells of very high homogeneity and purity show significant spectral heterogeneity. This heterogeneity is attributed to the fact that cells may be found at different stages of the reproductive cycle. Healthy and fully mature cells may not divide at all, whereas cells of the proliferative layer of epithelium may undergo slow division. In cancer cells, on the other hand, the division cycle may be a continuous process that leads to a rapid growth of the cancer. Cancerous
21 cells separated according to their reproductive cycle stages (*i.e.*, G1, S, G2 or M) show spectral patterns (Figure 4) that are distinctly different from the patterns observed in normal samples. Interpretation of these patterns is of prime importance for the understanding of different spectral patterns observed for normal and diseased samples of tissues and cells. As such, the next section is the primary piece of intellectual property to be protected by this disclosure.

26 Figure 4, trace A shows infrared absorption spectra collected from an exponentially growing cell culture of myeloid leukemia (ML-1) cells, and cells fractionated into the cell cycle phases by elutriation. Identification of the cells in a given fraction was performed by fluorescence activated cell sorting (FACS) analysis.

The S phase spectra in Figure 4 (trace C) resembles that of exponentially growing cells in
31 that it exhibits strong vibrations due to DNA. The spectra of cells in the G1 and G2 phases are similar to each other, and very different from the S phase spectrum in the low frequency region.

1 The shape of the peaks around $1070\text{--}1100\text{ cm}^{-1}$ (the “nose at 1085 cm^{-1} ”) suggests that in G1 and G2 phases, mostly RNA is observed. The results for cells in the G1 and G2 phases confirmed the hypothesis of opaque DNA: Since cells in the G1 phase are diploid (2 copies of DNA), whereas cells in the G2 phase are quadruploid (4 copies of DNA), one would observe stronger DNA signals if the nuclear DNA was detectable. However, since cells in the G1 and G2 phases exhibit very similar spectral features that bear the signatures of RNA, it follows that the infrared spectra do not detect the DNA in the nucleus, but rather, the cytoplasmic RNA. The strong contributions of DNA in the S phase (Trace C and D), however, are due to the DNA transcription which requires that sections of DNA are unwound from the chromatin, and thus, may become partially transparent to and detectable by IR radiation.

11 e) Aspects of Spectral Differences between Normal and Abnormal Samples

When examining healthy and diseased single squamous cells, one observes a mixture of different cells of different stages of maturation. Consequently, one observes a large variety of different spectral traces from these cells. In order to detect spectral changes due to disease, it is advantageous to ignore spectra due to cells that differ by states of maturation or differentiation.

16 This separation can be accomplished by data analysis (*vide infra*); i.e., there is no need to physically separate the cells in the sample.

After all data resulting from different stages of maturity are eliminated from the analysis, one can arrive at a set of spectral traces of cells that are still proliferative. These are the most important for analysis since their progeny cells will carry the same diseased genes. Even among these cells, one detects spectral inhomogeneity that may be attributed to the different phases of the cell cycle. For squamous tissue, for example, the spectral patterns of the immature cells differ significantly between normal and abnormal states of health in some of the observable division phases. However, prior to interpreting these differences in terms of presence or absence of disease, the heterogeneity of the spectral patterns due to the cell’s reproductive cycle must be established.

26 The differences between cancerous and non-cancerous cells can be observed most easily in selected cell cycle phases, whereas other phases exhibit virtually indistinguishable traces. Figure 5 A, for example, shows a comparison between healthy and diseased single cell spectra that are virtually superimposable. The low nucleic acid / protein ratio of these spectra suggests that the cells are not dividing actively (G0 or G1 stages). However, reference spectra for the pure G0 phase have not been observed in the ML-1 cells reported above, since they cycle continuously and never reach

1 the inactive phase G0. In normal samples most cells should be found in G0, and even in cancerous cells, the majority of all cells should be in G0.

Figure 5 B shows the differences between normal and abnormal cells in a phase whose spectra resemble those observed for the middle of the S phase. The spectra differ enormously in the intensity of the peak at 1230 cm^{-1} to the peak at 1300 cm^{-1} . The former of these contains 6 protein and DNA, whereas the latter is nearly a pure protein peak. Thus, any increase of the $1230/1300\text{ cm}^{-1}$ peak intensity ratio is an indication of the visibility of the DNA. This is further confirmed by the appearance of a sharp DNA peak at 970 cm^{-1} in the cancerous samples. Comparing individual spectra from normal and abnormal samples, spectral pairs similar to the ones depicted in Figures 5A and 5B can be found. Each spectral pair is thought to arise from a given 11 phase in the cell cycle, and the spectra always differ in the amount of spectral contribution of DNA. This is an indication that normal and cancerous single cells in a number of different stages of their reproductive cycle, can be distinguished by their detectable DNA content.

The intensity ratio of the peaks at $1230/1300\text{ cm}^{-1}$, however, is not a uniformly applicable indicator of disease. We found that any cells actively involved in replication or transcription has 16 a higher visible DNA content than inactive cells. Thus, cell cycle dependent data of cells from various organs need to be collected to create a baseline of how much DNA contribution constitute an abnormality.

The following section describes how the information presented above can be used to screen exfoliated or biopsied cells, tissues or other biological entities for the occurrence of cancer, 21 precancerous aspects, or other biological or physical abnormalities. In particular, the logical steps required to proceed from sample collection to a valid and reliable diagnosis for the most preferred embodiment of the invention will be discussed.

Epithelial cell samples can be derived by scraping the surface of the epithelium with suitable devices such as brushes, spatulas, *etc.*, used presently to collect specimens. Samples from 26 internal organs can be obtained by thin needle aspiration, needle biopsies or surgical biopsies. Cellular components of body fluids (lymphocytes and leukocytes) can be isolated directly from these body fluids. Standard methods, such as digestion with collagenase to break up tissues into individual cells, is utilized to obtain single cells suitable for spectroscopic analysis. Other biological specimens such as proteins and fragments of DNA, RNA, or other molecules may be 31 obtained by methods very well known in the art.

Cells and other biological entities obtained in this fashion are expected to be quite heterogeneous: exfoliated cells, for example, may contain cells at different stages of maturation,

1 whereas lymphocytes and leukocytes may be found at different stages of maturation and
differentiation. Cells derived from tissue sections may contain endothelial cells (for example, from
blood vessels). This heterogeneity is expected and will not present a significant problem if the
spectral data are collected on a cell-by-cell basis, rather than an averaged spectral collection. An
earlier patent (*e.g.*, Zakim and Lord, US Patent # 5,733,739) reports the use of cell pellets of
6 unknown and variable composition to carry out cancer screening; however, the similarity of
spectra of all cells and tissues render such an approach difficult or impossible to implement with
any reliability.

The cells or other entities obtained as described above are treated to remove impurities due
to blood, lymph, mucus or other confounding constituents. Simple separation procedures are
11 useful to enhance the percentage of desirable entities for the investigation.

The experiments described above reveal that most pronounced differences between normal
and abnormal cells are observed when cells replicate their DNA. In fully mature and terminally
differentiated cells, this process no longer occurs; thus, it is advantageous to remove them or
reduce their numbers.

16 The collected and purified cells are fixed to prevent degradation of the samples. Flash
fixing by ethanol (15 sec, low temperature) produces samples of sufficient stability for
spectroscopic analysis. Longer exposure to ethanol may dissolve the cell membranes, may lead to
cell fusion and precipitation of cellular components. Subsequently, cells are transferred to one of
a number of different infrared transparent substrates for spectral analysis for the most preferred
21 embodiment of the invention.

The spectroscopic sample for the most preferred embodiment consists of a partial layer of
cells with good separation between the cells. For the most preferred embodiment of the invention,
for example, 10^4 cells, each occupying about $2 \times 10^{-4} \text{ mm}^2$ and distributed uniformly on an area of
 50 mm^2 , result in a sample partial layer with an occupation of about 5 % of the surface of the
26 substrate. Such a sample is suitable for analysis by the method of the invention, since the infrared
absorption spectrum of each cell alone may be recorded. The samples reported by Zakim and
Lord often had sample populations 1000-10000 fold higher. The high population creates non-linear
absorption effects, retains the solvent in a manner that cannot be controlled, and may be
responsible for many of the artifacts reported by them. For the method of the present invention,
31 the spectrum of mostly single entities should be recorded. Overlapping a cancerous with a non
cancerous cell, for example, would lead to a spectrum which is not definitive for either type of cell.

1 Cells are most preferably visualized by staining with fluorescent, monatomic dyes (for example, isotonic Eu^{3+} ion solution). Such staining does not perturb the infrared spectra within the limits of detect ability, but permits the cells to be detected using fluorescence excitation. Identification of cells is necessary to avoid data collection from cell debris. An image of a typical cell sample under fluorescence excitation in the most preferred embodiment of the invention is
6 shown in Figure 6 which demonstrates the excellent separation of cells in this sample preparation procedure.

The area of the substrate (ca. 50 mm^2) that contains the cells is imaged using fluorescent and infrared wavelengths as follows. The substrate is inserted in the focal position 70 of a combination infrared microspectrometer (also known as an infrared microscope) and fluorescence
11 microscope shown schematically in figure 7. The infrared microspectrometer is equipped with a confocal, infrared-sensitive array detector 71 of 256×256 (=65536) individual diode elements on an area of about $7 \times 7 \text{ mm}^2$. Such instrumentation is commercially available. Fig. 7 shows is the optical arrangement for the infrared/fluorescent microscope combination which is the most preferred embodiment of the invention. Infrared light from the interferometer is passed through a
16 Cassegrain condenser 73A into the sample, collected via an identical Cassegrain objective 73B, and focused with an infrared transmitting lens such as a ZnSe lens 77 onto the confocal array detector 71. Reflecting optics could be used as well in place of the ZnSe lens. In order to reduce computation time for the analysis of up to 10,000 individual cells, only pixels that contain cellular spectral information is processed. This information is obtained by imaging the sample via
21 fluorescence microscopy prior to infrared data acquisition. To this end, the cells are illuminated by introducing an ultraviolet beam of light 74 from a UV lamp 75 into the optical train using a movable mirror 78. The UV light induces intense fluorescence from the cells in the focal region 70 that have been treated with Eu^{3+} ions. Since this stain consists of monatomic ions, it does not exhibit an infrared absorption spectrum; consequently, there is no spectral difference in the infrared
26 spectrum due to stained and unstained cells. The fluorescent light collected in the reflection mode as shown in fig. 7 is filtered to remove the excitation wavelength by a movable dichroic mirror 75, and is processed via a CCD detector 76. This image taken by the fluorescence light will indicate, by the fluorescent intensities, the position of cells on the sample substrate (cf. Figure 6). From these positions, one can identify which pixels of the confocal infrared array detector 71 need to
31 be processed to obtain the desired infrared spectral information of the individual cells. Clearly, the UV fluorescent image may equally well be taken in transmission mode, and/or the spectra of the

1 cells may be taken (at less resolution) in reflection mode after reflection from an IR reflecting substrate holding the cells

The analysis of the cells on the substrate proceeds as follows. The sample substrate is first illuminated with UV light that is absorbed by the Eu^{3+} stain with which the cells have been treated.

6 The fluorescence induced by the UV excitation is observed in reflection mode of the microscope, and collected through selective bandpass filters to reject the excitation wavelength. The fluorescence of the stained cells is detected by the CCD camera, and the positions of all cells on the substrate is computed by the positions of the pixels of the CCD camera detecting the fluorescent radiation.

Subsequently, the illumination of the sample is switched to infrared radiation. The
11 movable mirrors 75 and 78 are removed from the optical path of the microscope and the infrared light is passed through a step-scanning interferometer (not shown) to modulate the wavelength patterns of the light prior to being directed into the microscope. In the alternative, the movable mirror 78 and the dichroic mirror 75 could be formed on IR transparent substrates to reflect the appropriate light and transmit the infrared light. Each of the detector elements of the confocal
16 infrared array detector 71 is exposed to the infrared radiation passing through the sample cells which are imaged 1:1 onto the detector resulting in about 1 cell or less per detector element.

Interferograms are collected for all 65536 detector elements, and converted to infrared absorption spectra *via* a mathematical process known as Fourier transform (FT). Only the interferograms from detector pixels onto which cells are imaged are Fourier transformed to save
21 data analysis time. These pixels are identified by the fluorescence picture obtained in the previous step. Selecting only picture elements that are known to contain spectral information reduces the number of individual spectra to be calculated from the interferograms from 65000 to between 10,000 and 20,000.

The resulting spectra are uniformly expanded, smoothed and corrected for water vapor
26 absorption. Subsequently, the spectra (or their derivative spectra) are clustered using vector correlation methods. The clustering reveals the degree of “relatedness” of spectral patterns, and will reduce the number of independent observations to a few dozen spectral patterns for each sample. After this clustering, certain spectral traces that are clearly associated with cells of low information content are discarded. For example, spectra from red blood cells or fully mature,
31 inactive cells may be discarded. The resultant spectral patterns, referred henceforth as the “reduced spectral set” is analyzed as follows.

1 First, the reduced spectral set is compared to the cell cycle dependent reference sets that have been collected beforehand for the organ sample under investigation. This analysis reveals whether or not the distribution of the cell cycle stages falls within normal limits. Subsequently, all spectra in the reduced data set are searched for the occurrence of spectral patterns associated with a fast growing and rapidly dividing cell. The occurrence of such cells in a sample is indicative of
6 abnormality.

The analysis described above requires knowledge of the cellular spectra, and cellular distribution, observed for normal samples. The analysis, however, depends to a lesser degree on bases (reference) sets than the one described by Zakim and Lord, which used spectral averages over large number of cells. It is clear that such an averaging process reduces the sensitivity of the
11 method enormously, since only a small number of cells exhibit spectral traces modified by disease.

The method presented here uses both a statistical analysis of the spectral patterns of the individual cells (*i.e.*, changes in the distribution of cells at various stages of their development), as well as variations in the spectral patterns due to disease, for a vastly enhances sensitivity of the infrared spectral method.

16 A preferred embodiment of the invention is sketched in Fig. 8. A specimen generator 80 produces a plurality of specimen 82 in the same fashion as a FACS machine or an ink jet printer. The specimen 82 may be droplets containing the entities to be measured, or may be cells or other biological entities. The specimen are produced and fly through an atmosphere with a defined velocity. The atmosphere may be ordinary laboratory air, or may be air with a controlled water
21 vapor pressure or partial pressure of another vapor such as alcohol, or may be an inert gas such as nitrogen or argon, or vacuum. The specimen are generally electrically charged so that the specimen generator may accelerate them to a relatively high speed. However, such electrical charging is optional. The specimen are optionally marked with a fluorescent marker as detailed above for the marking of cells, and UV light source 81 and fluorescence detector 83 may be used to mark the
26 position of a specimen 82, or indeed may be used to decide whether specimen 82 contains entities to be investigated. The position and speed of each specimen is now known. A vibrational spectrum recording apparatus is now used to record the spectrum of each specimen, and the resulting plurality of spectra is treated as detailed above to characterize the statistics of all the specimen. In the present embodiment, the preferred vibrational spectrum recording device is two
31 infrared light sources 84 and 85 which direct infrared light on to a specimen 82C, and two detectors 86 and 87 which measure the light transmitted through specimen 82C. Light sources 84 and 85 are preferably pulsed laser sources which produce infrared light at frequencies characterizing the

1 infrared absorption spectrum of DNA and RNA respectively. A plurality of such lasers may be used to produce an entire vibrational spectrum for each specimen as detailed in the most preferred embodiment. A computer 88 is shown controlling specimen generator 80 and infrared sources 84 and 85, and monitoring fluorescence detector 83. Lines indicating control and monitor functions for light source 81 and detectors 86 and 87 are not shown to avoid complexity in fig. 8.

6 A preferred embodiment of the invention is shown in figure 9, where the separate infrared light sources 84 and 85 are replaced by a broad band infrared light source 94. The infrared light from the source 94 which is preferably a pulsed broad band laser, is focused on a specimen 82C which has been marked as noted above for figure 8. The broadband infrared light is then analyzed spectrally as indicated in fig. 9 by a focusing grating 96 which images light transmitted through the
11 cell onto an array detector 98 according to the infrared wavelength. Rays of two different infrared wavelengths are traced in fig. 9 in order to guide the eye. The grating 96 could be replaced by an imaging system used to image the light from the specimen 82C on to the slit of a normal spectrometer and infrared recording array detector, or indeed by any other spectral recording device as known in the spectroscopic art.

16 Lenses conventionally shown in fig. 8 and 9 are figurative only, and imaging and light handling devices as known in the art are anticipated by the inventors.

The vibrational spectrum recording devices of the invention are not limited to the devices described above, but may be any vibrational spectrum recording devices known in the spectroscopic art to record IR absorption spectra or Raman spectra.

21 Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that, withing the scope of the appended claims, the invention may be practiced otherwise then as specifically described.

1 1. A method of characterizing a biological specimen, comprising:

2 a) grouping a very large first plurality of entities into a second plurality of groups, each group

3 comprising a small number of entities;

4 b) characterizing each group of entities in the second plurality according to an aspect of the

5 vibrational spectrum of each group; and

6 c) statistically analyzing the characteristics of the groups of entities in the second plurality.

1 2. The method of claim 1, wherein the small number is preponderantly one.

1 3. The method of claim 1, wherein the entities are cells.

1 4. The method of claim 1, wherein characterization of each group is the recording of infrared

2 absorption spectra of the entities in each group.

1 5. The method of claim 4, wherein the small number is preponderantly one, and wherein the

2 entities are cells, and wherein the infrared absorption spectrum of each cell is analyzed for

3 indications that the one cell in each group is in a cell division stage.

1 6. The method of claim 5, wherein the results of the statistical analysis is the percentage of the cells

2 in the cell division stage.

1 7. The method of claim 5, wherein the indication that a cell is in a cell division stage is the

2 presence of a signal indicating DNA in the infrared absorption spectra.

1 8. The method of claim 4, wherein the small number is preponderantly one, and wherein entities

2 are grouped according to the fluorescence of the entities in each group.

- 1 9. A microscope, comprising:
- 2 infrared optics for imaging infrared light transmitted through a large number of entities on an area
- 3 of a microscope stage on to a first detector, where the first detector is an infrared array
- 4 detector; and
- 5 optics for imaging fluorescence light emitted by the entities on to a second detector, where the
- 6 second detector is a fluorescence light array detector.
- 1 10. The microscope of claim 9, further comprising:
- 2 a first source of infrared light, the infrared light for illuminating the area of the stage;
- 3 an second source of ultraviolet light, the ultraviolet light for illuminating the area of the stage.
- 1 11. The microscope of claim 10, wherein:
- 2 the first detector is an infra-red area array detector for detecting an image of the entities formed
- 3 by the infrared light transmitted through the entities;
- 4 and the second detector is an area array detector for detecting an image of the entities formed by
- 5 the fluorescence light emitted by the entities.
- 1 12. The microscope of claim 11, wherein the entities are single cells.
- 1 13. The microscope of claim 12, wherein the infrared absorption spectra of each cell is recorded.
- 1 14. The microscope of claim 13, wherein the infrared absorption spectrum of each cell is analyzed
- 2 for indications that the cell is in a cell division stage.
- 1 15. The microscope of claim 14, wherein the percentage of the cells in the cell division stage is
- 2 calculated.

1 16. The microscope of claim 14, wherein the indication that a cell is in a cell division stage is the
2 presence of a signal indicating DNA in the infrared absorption spectra.

1 17. An apparatus, comprising:

2 location means for locating a very large number of cells;

3 vibrational spectrum characterization means for characterizing the vibrational spectrum of each of
4 the cells located by the location means.

1 18. The apparatus of claim 17, wherein the vibrational spectrum characterization means comprises
2 a means for generating and for transmitting infrared light through each cell.

1 19. The apparatus of claim 18, wherein the means for generating infrared light comprises a first
2 laser having a first defined infrared wavelength.

1 20. The apparatus of claim 19, wherein the first laser is pulsed when the location means locates a
2 first cell in a position to be characterized by the first laser.

1 21. The apparatus of claim 19, wherein the first defined wavelength comprises a wavelength
2 wherein DNA is highly absorbing.

1 22. The apparatus of claim 21, wherein a second laser having a second infrared wavelength is
2 pulsed to characterize the first cell, wherein the second infrared wavelength comprises a
3 wavelength wherein RNA is highly absorbing

1 23. The apparatus of claim 20, wherein the first defined wavelength comprises a wavelength
2 wherein DNA is highly absorbing.

1 24. The apparatus of claim 18, wherein the means for generating infrared light comprises a third
2 laser having a broad band infrared wavelength range.

- 1 25. The apparatus of claim 24, wherein the third laser is pulsed when the location means locates
2 a first cell in a position to be characterized by the laser.
- 1 26. The apparatus of claim 25, wherein the broad band infrared wavelength range includes a
2 wavelength wherein DNA is highly absorbing.
- 1 27. The apparatus of claim 26, wherein the broad band infrared wavelength range includes a
2 wavelength wherein RNA is highly absorbing.
- 1 28. The apparatus of claim 27, wherein the infrared absorption spectrum of each cell is recorded.
- 1 29. The apparatus of claim 28, wherein the infrared absorption spectrum of each cell is analyzed
2 for indications that the cell is in a cell division stage.
- 1 30. The apparatus of claim 29, wherein the percentage of the cells in the cell division stage is
2 calculated.
- 1 31. The apparatus of claim 30, wherein the indication that a cell is in a cell division stage is the
2 presence of a signal indicating DNA in the infrared absorption spectra.
- 1 33. The apparatus of claim 17, wherein the location means is a fluorescence activated sorting
2 apparatus
- 1 34. A method of characterizing a large group of biological cells, comprising:
- 2 a) separating the cells so that the cells of the large group are preponderantly separated from each
3 other;
- 4 b) characterizing each cell according to an aspect of the vibrational spectrum each cell; and
- 5 c) statistically analyzing the characteristics of the groups cells.

1 39. The method of claim 38, wherein the separated cells are located according to the fluorescence
2 of the cells.

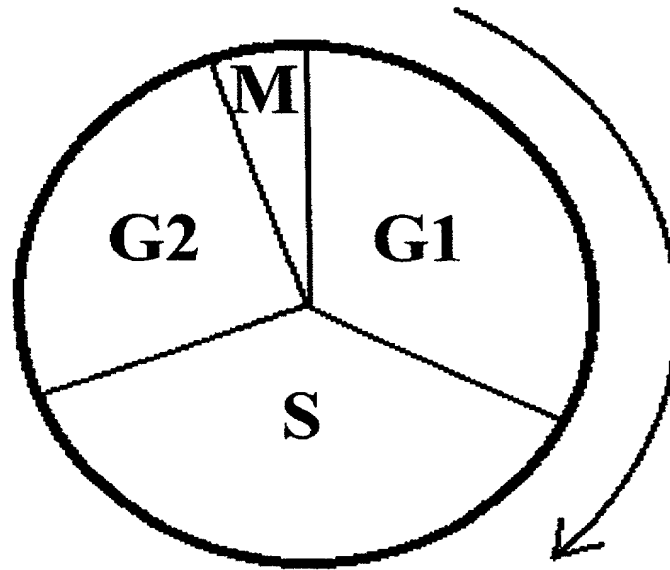
Method of Characterization of Biological Entities

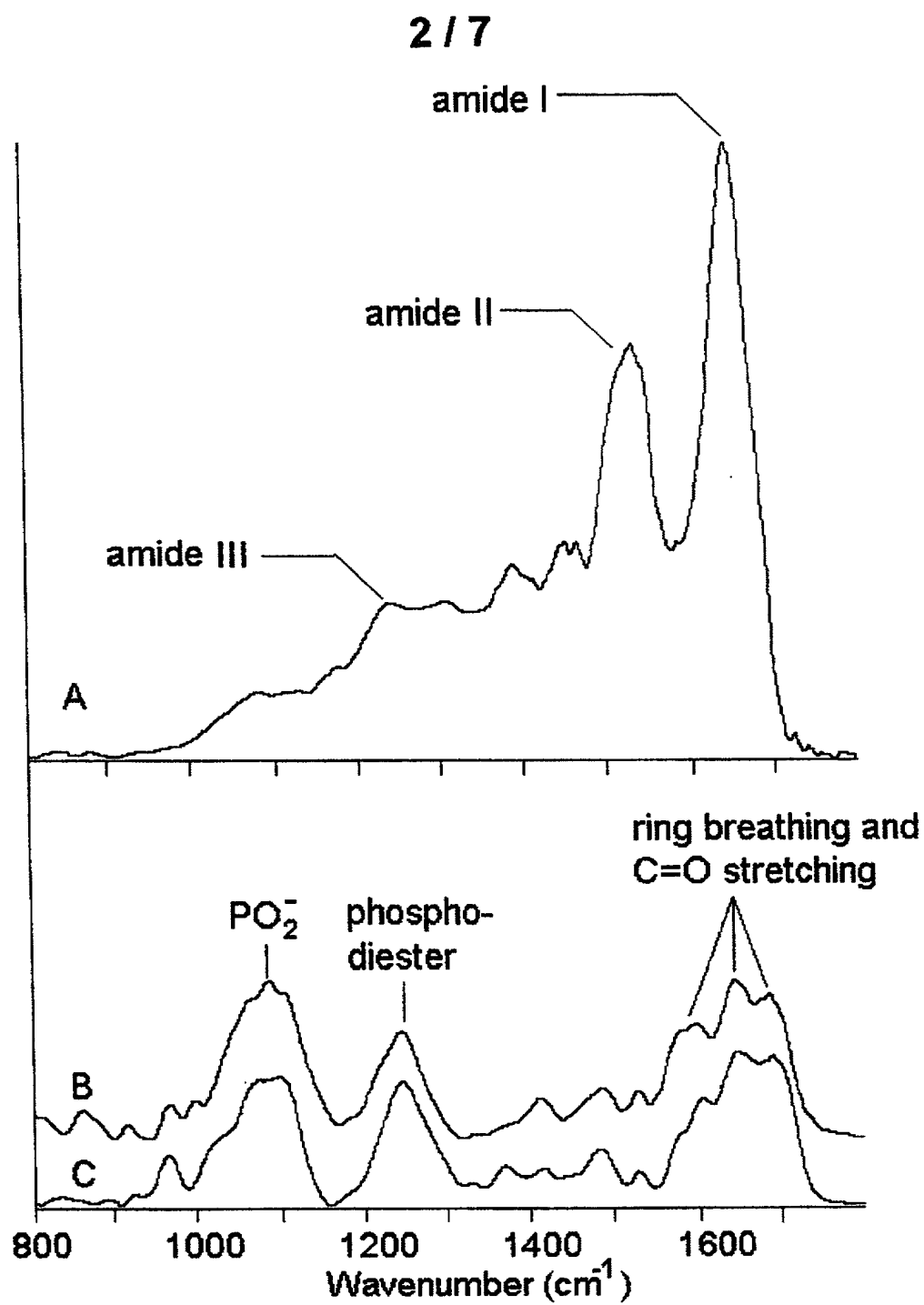
ABSTRACT OF THE INVENTION

An apparatus and method are disclosed for measuring the infrared vibrational spectral characteristics of each of a large number of biological entities such as cells, and from the measurements statistically determining the presence of anomalies such as cancer.

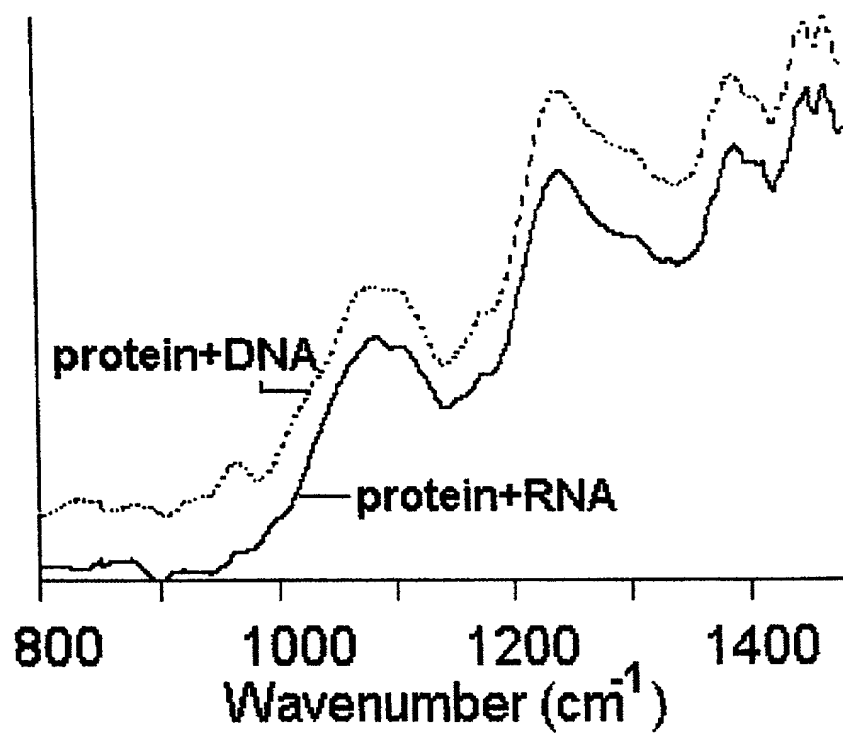
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000
1001
1002
1003
1004
1005
1006
1007
1008
1009
1010
1011
1012
1013
1014
1015
1016
1017
1018
1019
1020
1021
1022
1023
1024
1025
1026
1027
1028
1029
1030
1031
1032
1033
1034
1035
1036
1037
1038
1039
1040
1041
1042
1043
1044
1045
1046
1047
1048
1049
1050
1051
1052
1053
1054
1055
1056
1057
1058
1059
1060
1061
1062
1063
1064
1065
1066
1067
1068
1069
1070
1071
1072
1073
1074
1075
1076
1077
1078
1079
1080
1081
1082
1083
1084
1085
1086
1087
1088
1089
1090
1091
1092
1093
1094
1095
1096
1097
1098
1099
1100
1101
1102
1103
1104
1105
1106
1107
1108
1109
1110
1111
1112
1113
1114
1115
1116
1117
1118
1119
1120
1121
1122
1123
1124
1125
1126
1127
1128
1129
1130
1131
1132
1133
1134
1135
1136
1137
1138
1139
1140
1141
1142
1143
1144
1145
1146
1147
1148
1149
1150
1151
1152
1153
1154
1155
1156
1157
1158
1159
1160
1161
1162
1163
1164
1165
1166
1167
1168
1169
1170
1171
1172
1173
1174
1175
1176
1177
1178
1179
1180
1181
1182
1183
1184
1185
1186
1187
1188
1189
1190
1191
1192
1193
1194
1195
1196
1197
1198
1199
1200
1201
1202
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
1213
1214
1215
1216
1217
1218
1219
1220
1221
1222
1223
1224
1225
1226
1227
1228
1229
1230
1231
1232
1233
1234
1235
1236
1237
1238
1239
1240
1241
1242
1243
1244
1245
1246
1247
1248
1249
1250
1251
1252
1253
1254
1255
1256
1257
1258
1259
1260
1261
1262
1263
1264
1265
1266
1267
1268
1269
1270
1271
1272
1273
1274
1275
1276
1277
1278
1279
1280
1281
1282
1283
1284
1285
1286
1287
1288
1289
1290
1291
1292
1293
1294
1295
1296
1297
1298
1299
1300
1301
1302
1303
1304
1305
1306
1307
1308
1309
1310
1311
1312
1313
1314
1315
1316
1317
1318
1319
1320
1321
1322
1323
1324
1325
1326
1327
1328
1329
1330
1331
1332
1333
1334
1335
1336
1337
1338
1339
1340
1341
1342
1343
1344
1345
1346
1347
1348
1349
1350
1351
1352
1353
1354
1355
1356
1357
1358
1359
1360
1361
1362
1363
1364
1365
1366
1367
1368
1369
1370
1371
1372
1373
1374
1375
1376
1377
1378
1379
1380
1381
1382
1383
1384
1385
1386
1387
1388
1389
1390
1391
1392
1393
1394
1395
1396
1397
1398
1399
1400
1401
1402
1403
1404
1405
1406
1407
1408
1409
1410
1411
1412
1413
1414
1415
1416
1417
1418
1419
1420
1421
1422
1423
1424
1425
1426
1427
1428
1429
1430
1431
1432
1433
1434
1435
1436
1437
1438
1439
1440
1441
1442
1443
1444
1445
1446
1447
1448
1449
1450
1451
1452
1453
1454
1455
1456
1457
1458
1459
1460
1461
1462
1463
1464
1465
1466
1467
1468
1469
1470
1471
1472
1473
1474
1475
1476
1477
1478
1479
1480
1481
1482
1483
1484
1485
1486
1487
1488
1489
1490
1491
1492
1493
1494
1495
1496
1497
1498
1499
1500
1501
1502
1503
1504
1505
1506
1507
1508
1509
1510
1511
1512
1513
1514
1515
1516
1517
1518
1519
1520
1521
1522
1523
1524
1525
1526
1527
1528
1529
1530
1531
1532
1533
1534
1535
1536
1537
1538
1539
1540
1541
1542
1543
1544
1545
1546
1547
1548
1549
1550
1551
1552
1553
1554
1555
1556
1557
1558
1559
1560
1561
1562
1563
1564
1565
1566
1567
1568
1569
1570
1571
1572
1573
1574
1575
1576
1577
1578
1579
1580
1581
1582
1583
1584
1585
1586
1587
1588
1589
1590
1591
1592
1593
1594
1595
1596
1597
1598
1599
1600
1601
1602
1603
1604
1605
1606
1607
1608
1609
1610
1611
1612
1613
1614
1615
1616
1617
1618
1619
1620
1621
1622
1623
1624
1625
1626
1627
1628
1629
1630
1631
1632
1633
1634
1635
1636
1637
1638
1639
1640
1641
1642
1643
1644
1645
1646
1647
1648
1649
1650
1651
1652
1653
1654
1655
1656
1657
1658
1659
1660
1661
1662
1663
1664
1665
1666
1667
1668
1669
1670
1671
1672
1673
1674
1675
1676
1677
1678
1679
1680
1681
1682
1683
1684
1685
1686
1687
1688
1689
1690
1691
1692
1693
1694
1695
1696
1697
1698
1699
1700
1701
1702
1703
1704
1705
1706
1707
1708
1709
1710
1711
1712
1713
1714
1715
1716
1717
1718
1719
1720
1721
1722
1723
1724
1725
1726
1727
1728
1729
1730
1731
1732
1733
1734
1735
1736
1737
1738
1739
1740
1741
1742
1743
1744
1745
1746
1747
1748
1749
1750
1751
1752
1753
1754
1755
1756
1757
1758
1759
1760
1761
1762
1763
1764
1765
1766
1767
1768
1769
1770
1771
1772
1773
1774
1775
1776
1777
1778
1779
1780
1781
1782
1783
1784
1785
1786
1787
1788
1789
1790
1791
1792
1793
1794
1795
1796
1797
1798
1799
1800
1801
1802
1803
1804
1805
1806
1807
1808
1809
1810
1811
1812
1813
1814
1815
1816
1817
1818
1819
1820
1821
1822
1823
1824
1825
1826
1827
1828
1829
1830
1831
1832
1833
1834
1835
1836
1837
1838
1839
1840
1841
1842
1843
1844
1845
1846
1847
1848
1849
1850
1851
1852
1853
1854
1855
1856
1857
1858
1859
1860
1861
1862
1863
1864
1865
1866
1867
1868
1869
1870
1871
1872
1873
1874
1875
1876
1877
1878
1879
1880
1881
1882
1883
1884
1885
1886
1887
1888
1889
1890
1891
1892
1893
1894
1895
1896
1897
1898
1899
1900
1901
1902
1903
1904
1905
1906
1907
1908
1909
1910
1911
1912
1913
1914
1915
1916
1917
1918
1919
1920
1921
1922
1923
1924
1925
1926
1927
1928
1929
1930
1931
1932
1933
1934
1935
1936
1937
1938
1939
1940
1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
1951
1952
1953
1954
1955
1956
1957
1958
1959
1960
1961
1962
1963
1964
1965
1966
1967
1968
1969
1970
1971
1972
1973
1974
1975
1976
1977
1978
1979
1980
1981
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
1993
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2022
2023
2024
2025
2026
2027
2028
2029
2030
2031
2032
2033
2034
2035
2036
2037
2038
2039
2040
2041
2042
2043
2044
2045
2046
2047
2048
2049
2050
2051
2052
2053
2054
2055
2056
2057
2058
2059
2060
2061
2062
2063
2064
2065
2066
2067
2068
2069
2070
2071
2072
2073
2074
2075
2076
2077
2078
2079
2080
2081
2082
2083
2084
2085
2086
2087
2088
2089
2090
2091
2092
2093
2094
2095
2096
2097
2098
2099
2100
2101
2102
2103
2104
2105
2106
2107
2108
2109
2110
2111
2112
2113
2114
2115
2116
2117
2118
2119
2120
2121
2122
2123
2124
2125
2126
2127
2128
2129
2130
2131
2132
2133
2134
2135
2136
2137
2138
2139
2140
2141
2142
2143
2144
2145
2146
2147
2148
2149
2150
2151
2152
2153
2154
2155
2156
2157
2158
2159
2160
2161
2162
2163
2164
2165
2166
2167
2168
2169
2170
2171
2172
2173
2174
2175
2176
2177
2178
2179
2180
2181
2182
2183
2184
2185
2186
2187
2188
2189
2190
2191
2192
2193
2194
2195
2196
2197
2198
2199
2200
2201
2202
2203
2204
2205
2206
2207
2208
2209
2210
2211

1 / 7

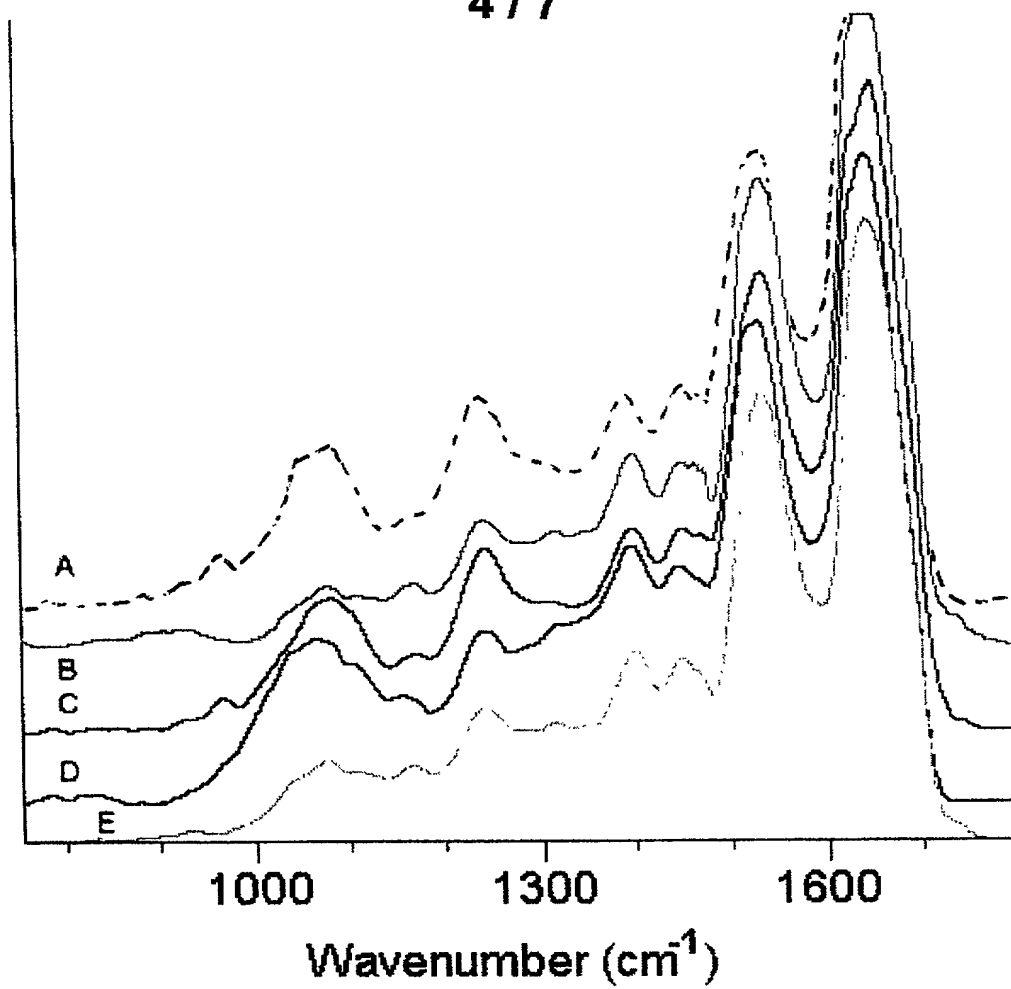
*Figure 1*

*Figure 2*

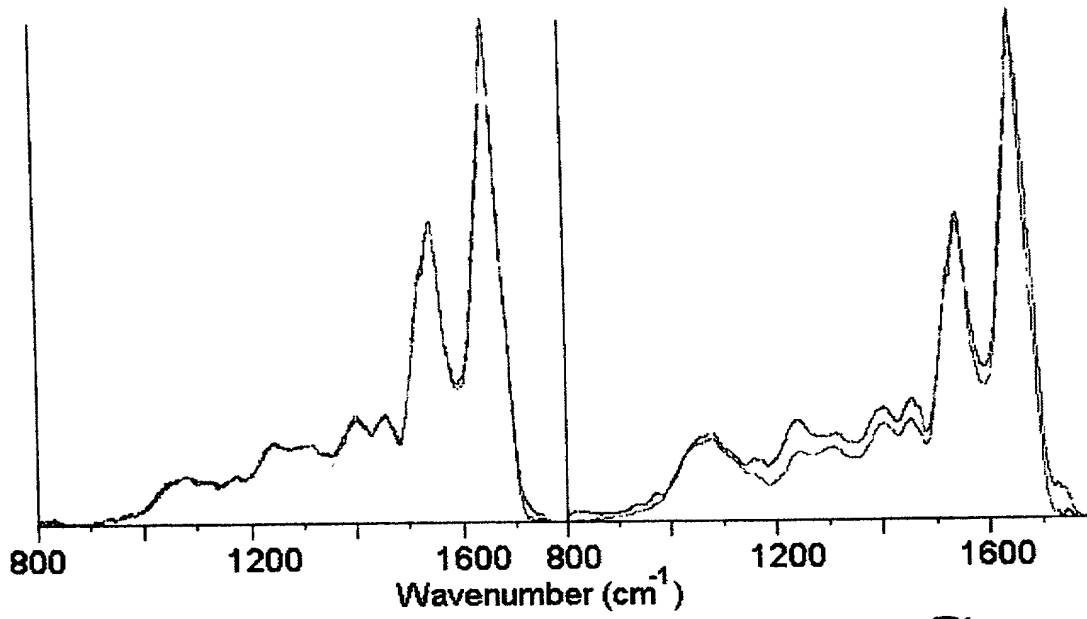
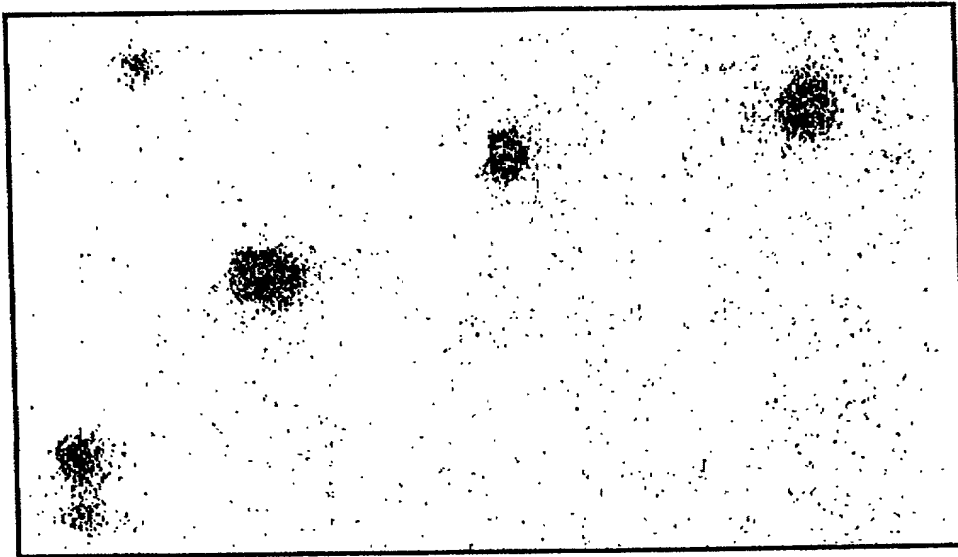
317

*Figure 3*

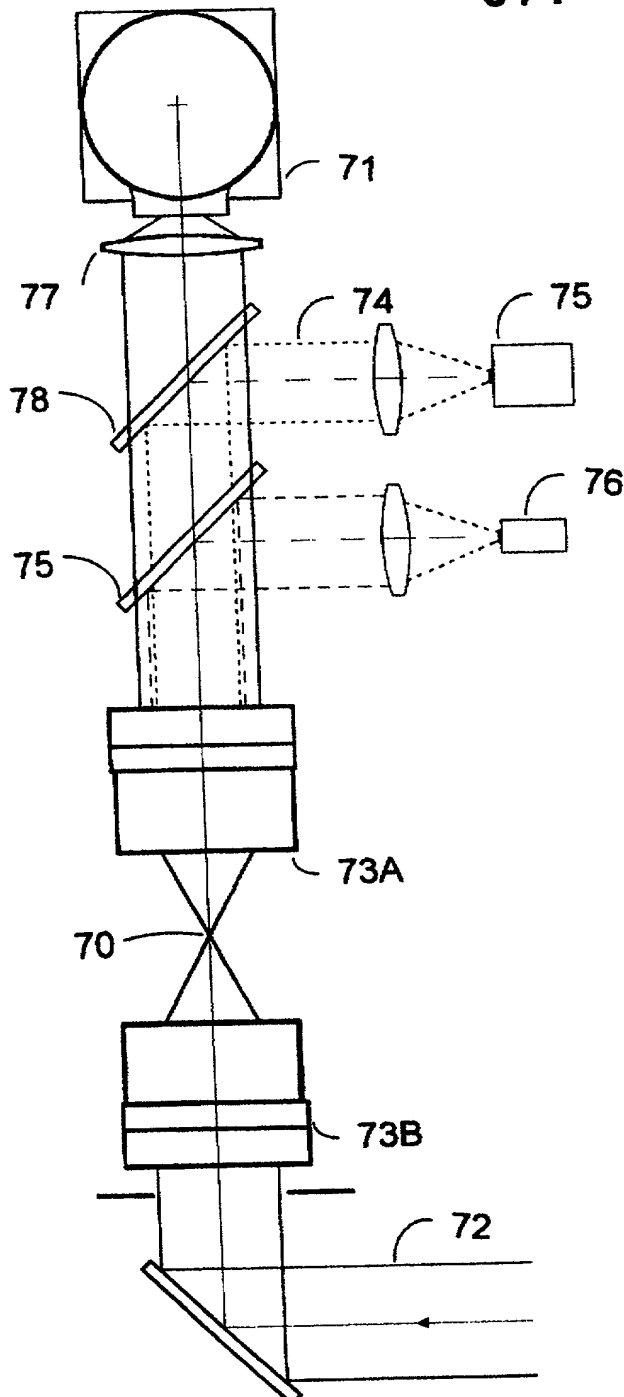
4/7

*Figure 4*

5/7

*Figure 5**Figure 6*

6 / 7



**Confocal HgCdTe
Array Detector**

ZnSe Lens

UV Lamp

CCD

**Cassegrain
Collector**

Sample Position

**Cassegrain
Condenser**

**Intensity Modulated
IR Beam from
Interferometer**

Figure 7

717

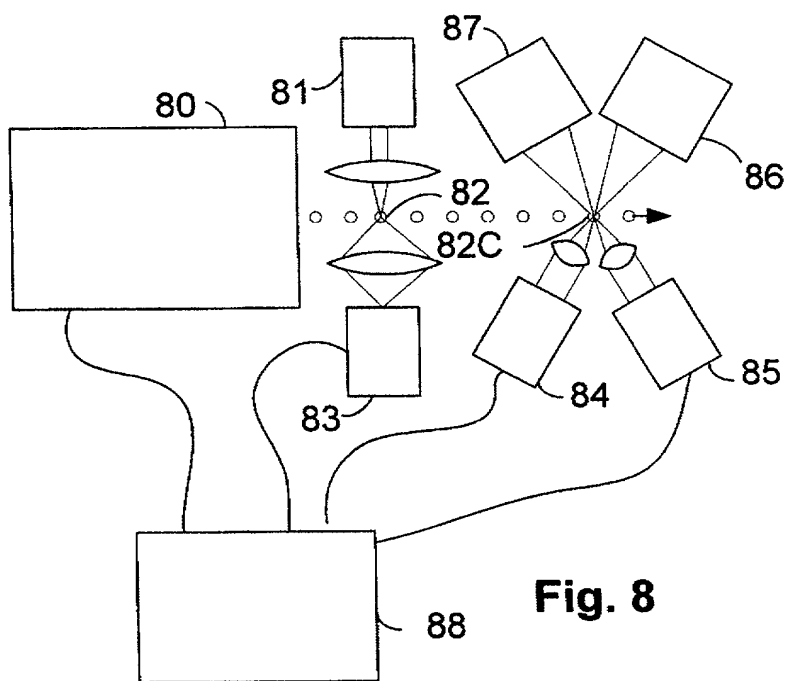


Fig. 8

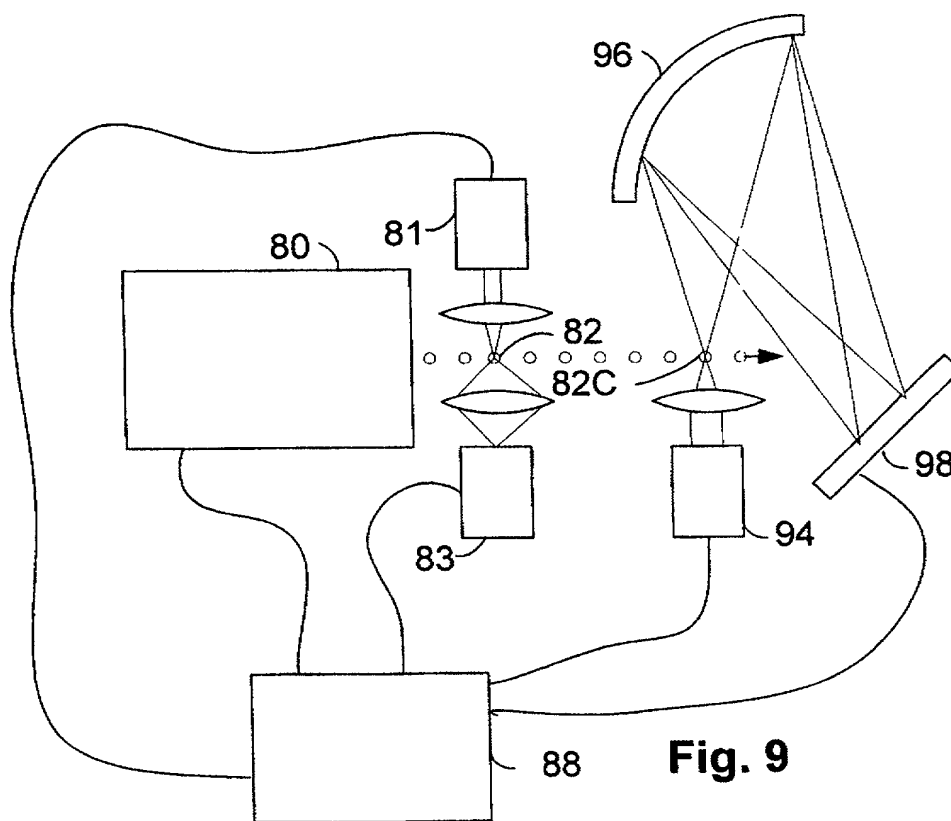


Fig. 9

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method of Characterization of Biological Entities

the specification of which (check one)

☒ is attached hereto.

☐ was filed on _____ as

Application Serial No.

and was amended on

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s) Priority Claimed

(Number)(Country) (Day/Month/Year Filed) Yes No

I hereby claim the benefit under Title 35, United States Code, Section 119(e) and/or Section 120 of any United States Provisional Application(s) and Nonprovisional Application(s) listed below and, insofar as the subject matter of each of the claims of the application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information material to the patentability of this application as defined in Title 37, Code of Federal Regulations, Section 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application;

60/33,529 1/22/98 pending
(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith (list name and registration number).

Rodney T. Hodgson, PhD. (Reg. No. 37,849); Ronald L. Drumheller, (Reg. No. 25,674); Sean Patrick Suiter, (Reg. No. 34260);

Send Correspondence to: Rodney T. Hodgson, PhD.

822 Pinesbridge Road, Ossining, NY 10562

Direct Telephone and/or FAX Calls to: Rodney T. Hodgson (914)762-5248 (FAX)(914) 762-4126

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

Method of Characterization of Biological Entities

Max Diem
Full name of first inventor

Max Diem
Inventor's Signature

11 Ackerman Court, Croton-on-Hudson, NY 10520
Residence and Post Office Address

Germany
Citizenship

Date

Jan 5, 2000

Jill Bargonetti
Full name of second joint-inventor,

Jill Bargonetti
Inventor's signature

Date

Jan 5, 2000

711 Amsterdam Ave Apt 12H, New York, NY 10025
Residence and Post Office Address

U.S.A.
Citizenship

Tamara Gopen
Full name of third joint inventor,

Tamara Gopen
Inventor's Signature

Date Jan 5, 2000

135 East 29th Street, Apt 27G, New York, NY 10016
Residence and Post Office Address

U.S.A.
Citizenship

Susie Boydston-White
Full name of fourth joint-inventor,

Susie Boydston-White
Inventor's Signature

Date 06 January 2000

338 East 67th Street Apt 6, New York, NY 10021
Residence and Post Office Address

U.S.A.
Citizenship